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## **Glucocorticoid sensitivity of circulating monocytes in essential hypertension.**

Wirtz, P H ; von Känel, R ; Frey, K ; Ehlert, Ulrike ; Fischer, J E

**Abstract:** BACKGROUND: Essential hypertension ranks among the strongest cardiovascular risk factors. Cytokine production by monocytes plays a key role in atherosclerosis development and acute coronary syndromes. We investigated whether stimulated monocyte cytokine release and its inhibition by glucocorticoids would differ between hypertensive and normotensive subjects. **METHODS:** Study participants were 222 middle-aged male employees with industrial jobs. Following the criteria of the World Health Organization/International Society for Hypertension, 76 subjects were classified as being hypertensive (systolic blood pressure  $\geq$  140 mm Hg or diastolic blood pressure  $\geq$  90 mm Hg). In vitro monocyte tumor necrosis factor (TNF)- $\alpha$  release after lipopolysaccharide (LPS) stimulation was assessed with and without coinubation with incremental doses of dexamethasone. Monocyte glucocorticoid sensitivity was defined as the dexamethasone concentration inhibiting TNF- $\alpha$  release by 50%. **RESULTS:** Hypertensive subjects showed 11% higher LPS-stimulated TNF- $\alpha$  release than normotensive subjects ( $F(1,181) = 5.21$ ,  $P = .024$ ). In hypertensive subjects, monocyte glucocorticoid sensitivity was 21% lower than in normotensive subjects ( $F(1,178) = 4.94$ ,  $P = .027$ ), indicating that dexamethasone inhibited relatively less TNF- $\alpha$  release in hypertensive subjects. Results held significance when a set of classic cardiovascular risk factors was controlled for. **CONCLUSION:** The findings suggest that proinflammatory activity of circulating monocytes is higher in hypertensive than in normotensive men, providing one potential pathway to explain the increased atherosclerotic risk with essential hypertension.

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# Glucocorticoid Sensitivity of Circulating Monocytes in Essential Hypertension

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Ulrike Ehlert, and Joachim E. Fischer

**Background:** Essential hypertension ranks among the strongest cardiovascular risk factors. Cytokine production by monocytes plays a key role in atherosclerosis development and acute coronary syndromes. We investigated whether stimulated monocyte cytokine release and its inhibition by glucocorticoids would differ between hypertensive and normotensive subjects.

**Methods:** Study participants were 222 middle-aged male employees with industrial jobs. Following the criteria of the World Health Organization/International Society for Hypertension, 76 subjects were classified as being hypertensive (systolic blood pressure  $\geq 140$  mm Hg or diastolic blood pressure  $\geq 90$  mm Hg). In vitro monocyte tumor necrosis factor (TNF)- $\alpha$  release after lipopolysaccharide (LPS) stimulation was assessed with and without coinubation with incremental doses of dexamethasone. Monocyte glucocorticoid sensitivity was defined as the dexamethasone concentration inhibiting TNF- $\alpha$  release by 50%.

**Results:** Hypertensive subjects showed 11% higher LPS-stimulated TNF- $\alpha$  release than normotensive subjects ( $F_{1,181} = 5.21$ ,  $P = .024$ ). In hypertensive subjects, monocyte glucocorticoid sensitivity was 21% lower than in normotensive subjects ( $F_{1,178} = 4.94$ ,  $P = .027$ ), indicating that dexamethasone inhibited relatively less TNF- $\alpha$  release in hypertensive subjects. Results held significance when a set of classic cardiovascular risk factors was controlled for.

**Conclusion:** The findings suggest that proinflammatory activity of circulating monocytes is higher in hypertensive than in normotensive men, providing one potential pathway to explain the increased atherosclerotic risk with essential hypertension. *Am J Hypertens* 2004;17: 489–494 © 2004 American Journal of Hypertension, Ltd.

**Key Words:** Essential hypertension, glucocorticoid sensitivity, monocytes, cytokines, cardiovascular disease.

**P**rimarily hypertension ranks among the leading risk factors for vascular diseases.<sup>1,2</sup> The mechanisms that link hypertension with increased incidence of atherosclerosis are not fully understood.<sup>3,4</sup> Monocytes play a key role in the pathogenesis and progression of atherosclerosis.<sup>5,6</sup> They secrete inflammatory cytokines that further promote inflammation in atherosclerotic lesions.<sup>5–8</sup> Elevated plasma levels of proinflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$  or interleukin (IL)-6 have been prospectively associated with increased cardiovascular risk in healthy individuals and in those with coronary artery disease.<sup>9–11</sup> Although this has not been investigated prospectively, exaggerated monocyte proinflammatory cytokine release might account for heightened cardiovascular risk in hypertension.

Regulatory processes in monocyte cytokine release entail stimulation of cytokine secretion as well as its inhibition. A potent stimulator of monocyte cytokine production is lipopolysaccharide (LPS), whereas glucocorticoids effectively down-regulate monocyte cytokine release.<sup>12–14</sup> In a previous study LPS-stimulated monocyte cytokine release has been examined in 22 hypertensive patients. In that study, peripheral blood monocytes of patients with essential hypertension secreted more TNF- $\alpha$  and IL-1 $\beta$  but not more IL-6 in response to LPS than did monocytes from normotensive control subjects.<sup>15</sup> As a whole, there is a paucity of investigation concerning regulation of human monocyte activity in essential hypertension.<sup>15</sup> Moreover, despite the fact that excess cortisol is known to elevate blood pressure (BP),<sup>16</sup> to the best of our knowledge,

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**Table 1.** Health factors of normotensive and hypertensive men

	Normotensive ( <i>n</i> = 146)	Hypertensive ( <i>n</i> = 76)	<i>P</i>
Systolic BP (mm Hg)	124 ± 0.67	146 ± 1.8	< .001
Diastolic BP (mm Hg)	77.1 ± 0.5	92.0 ± 1.2	< .001
Age (y)	37.9 ± 0.7	45.2 ± 0.9	< .001
Body mass index (kg/m <sup>2</sup> )	25.66 ± 0.2	27.5 ± 0.4	< .001
LDL/HDL ratio	2.89 ± 0.08	3.18 ± 0.11	.032
Hemoglobin Alc (%)	5.11 ± 0.03	5.28 ± 0.05	.005
Cigarettes/day	6.6 ± 0.8	5.2 ± 1.2	.331
Unstimulated TNF- $\alpha$ (pg/mL)	1.92 ± 0.15	1.88 ± 0.08	.853
Monocytes ( $\times 10^5$ /mL)	5.95 ± 0.14	5.83 ± 0.18	.605
Urinary cortisol ( $\mu$ g/L)	41.9 ± 2.1	46.9 ± 5.4	.306

Values given are mean  $\pm$  SEM.

BP = blood pressure; HDL = high-density lipoprotein cholesterol; LDL = low-density lipoprotein cholesterol.

down-regulation of monocyte cytokine release by glucocorticoids has not been investigated in hypertensive subjects.

We thus wondered whether suppression of the monocyte proinflammatory response by glucocorticoids (ie, monocyte glucocorticoid sensitivity) would be altered in hypertensive compared with normotensive individuals. Such a difference might provide one biologic mechanism linking hypertension with the pathogenesis of atherosclerosis. Following previous methods, we assessed monocyte reactivity by means of measuring TNF- $\alpha$  in whole blood after LPS stimulation.<sup>13,14</sup> We calculated the monocyte glucocorticoid sensitivity as the concentration of dexamethasone required to suppress LPS-stimulated TNF- $\alpha$  release by 50%.<sup>12,17,18</sup>

## Methods

### Study Population

The Institutional Review Board of the Swiss Federal Institute of Technology approved the study protocol and the process for obtaining informed consent. A representative sample of 647 men and women from a total of 1760 employees of an airplane manufacturing plant in Southern Germany were invited to participate in the study. Of these, 325 subjects (280 men and 45 women) volunteered to participate. Except for a slightly higher age, there were no baseline demographic differences between volunteers and nonparticipants (data not shown). For the present study, we considered only men to avoid hormonal confounding of inflammatory activity by oral contraceptives or by the female cycle. We excluded subjects who were on any antihypertensive medication (including  $\beta$ -blockers), hormone therapy, psychoactive drugs, or steroids. This procedure left 222 men, all of white ethnicity, whom we classified into hypertensive and normotensive individuals based on their BP following the World Health Organization/International Society for Hypertension definition (systolic BP  $\geq 140$  mm Hg or diastolic BP  $\geq 90$  mm Hg).<sup>19</sup> In the following sections, we present the comparisons be-

tween hypertensive (*n* = 76) and normotensive (*n* = 146) men. Table 1 lists characteristics of the study participants.

### Experimental Protocol

All subjects completed an extensive questionnaire with respect to personal medical history, cardiovascular risk factors, intake of medications, and medical history of the participants' mother and father as previously described.<sup>20,21</sup> None of the individuals with hypertension were on any medication to reduce BP, and only two of the hypertensive subjects were aware of their condition. Thereafter, subjects had a 15-min rest period while sitting, followed by BP determination twice within 5 min using a mercury sphygmomanometer. All subjects also had their weight and height measured to compute the body mass index (BMI).

Within 3 weeks, all participants were rescheduled on a workday and 2 h after awakening (between 7:00 AM and 8:45 AM) for collection of a fasting blood sample. Assays were started within 5 min from blood collection in a cell culture facility adjacent to the blood collection room. For the TNF- $\alpha$  assay, blood was processed by standard techniques using cooled (4°C) citrated tubes. Urine collection for overnight cortisol secretion started at 9:00 PM the night before blood sampling, ending with the inclusion of the first void after awakening.<sup>22</sup>

### Glucocorticoid Sensitivity and Assay

Monocytes are the main cytokine producing cells in LPS-stimulated whole blood.<sup>23</sup> To assess glucocorticoid sensitivity of stimulated cytokine production, dexamethasone has been widely used.<sup>12,14,24</sup> Because of its association with atherosclerosis,<sup>5,25</sup> we chose to study TNF- $\alpha$  plasma concentrations. TNF- $\alpha$  plays a crucial role in the initial activation of inflammatory changes such as stimulation of C-reactive protein production by the liver, which is thought to sustain atherosclerosis development.<sup>6</sup>

The whole blood cell culture is an *in vitro* method to analyze cytokine secretion in a controlled environment as

well as to study the biologic effects of drugs on cytokine release.<sup>26,27</sup> The whole blood assay avoids possible biases through artificial stimulation of monocytes arising from preanalytical steps by mononuclear cell separation, and it also preserves the “natural environment” (including hormones) of cytokine producing cells.<sup>27,28</sup>

The basic principle of the glucocorticoid sensitivity assay is to use a stimulant for cytokine release coincubated with particular concentrations of a glucocorticoid. After a defined incubation period, the cytokine content of the supernatant is determined. If a stimulant such as LPS is used that predominantly activates monocytes, and if a cytokine such as TNF- $\alpha$  is assessed whose main source in LPS-stimulated whole blood is monocytes, one may be confident that the assay mainly reflects monocyte regulation.

The assay provides two major endpoints: a) the cytokine release in response to LPS (without glucocorticoid costimulation), and b) the estimated glucocorticoid concentration that would exactly inhibit 50% of the LPS-stimulated cytokine release determined from (a). Measure (b) is independent from the absolute cytokine release, and it has been referred to as the IC<sub>50</sub>, which is a single-measure index to describe the glucocorticoid sensitivity. To estimate IC<sub>50</sub> values from the inhibition curve, we used a logistic function.

In detail, the method was as follows. Venous blood was collected in heparinized tubes, diluted 1:10 with saline. The blood was then incubated with LPS (*Escherichia coli*, 055:B5, no. L2880; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and five different concentrations of dexamethasone (no. D8893, Sigma-Aldrich Chemie), both dissolved in sterile saline solution (NaCl 0.9%, Fresenius Kabi, Stans, Switzerland), on a 24-well plate (no. 3047; Becton Dickinson, San Diego, CA). Diluted whole blood (400  $\mu$ L) was added to 50  $\mu$ L of LPS and to 50  $\mu$ L of various concentrations of dexamethasone. Final concentrations on the plate were 15 ng/mL LPS and 0, 10<sup>-10</sup>, 10<sup>-9</sup>, 10<sup>-8</sup>, and 10<sup>-7</sup> mol/L dexamethasone. After a 6-h incubation period at 37°C in 5% CO<sub>2</sub>, plates were centrifuged for 10 min at 2000 g at 4°C.<sup>12,14</sup> The supernatant was collected and stored at -80°C until assayed.

## Biochemical Analyses

In vitro levels of TNF- $\alpha$  were determined using commercial ELISA kits (BD Pharmingen, San Diego, CA). A high-sensitivity assay was chosen to measure in vivo plasma levels of TNF- $\alpha$  (ELISA; Quantikine HS, R&D Systems Europe, Abington, UK). Low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, hemoglobin A1c (HbA1c), and urinary cortisol were determined by a commercial laboratory (Synlab, Augsburg, Germany) by means of standard procedures. Monocyte counts were determined from EDTA samples within 3 h from blood sampling using the SE-9000 cell counter (Sysmex, Norderstedt, Germany).

## Statistical Analyses

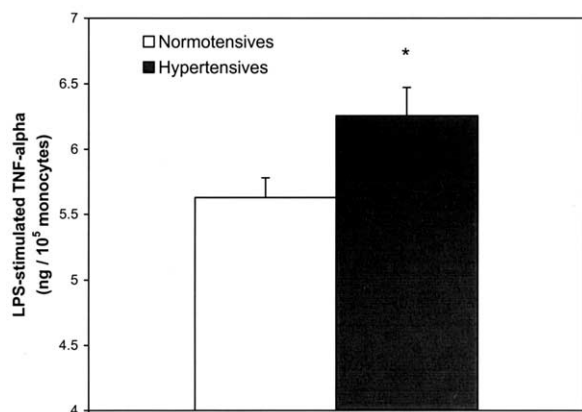
All calculations were performed using SPSS version 10.0 (SPSS, Chicago, IL) and Curve Expert version 1.3 software packages (Shareware, Daniel Hyams, Starkville, MS). Data are presented as mean  $\pm$  SEM. Results were considered statistically significant at the level of  $P < .05$ , and all tests were two-tailed. In case of missing data, cases were excluded listwise. Across the two subject groups, univariate analyses of variance (ANOVAs) were calculated for health factors (Table 1). Univariate analyses of covariance were computed for LPS-stimulated cytokine production, and IC<sub>50</sub> values with the following established cardiovascular risk factors as covariates: age, BMI, HbA1c, LDL/HDL ratio, systolic BP, diastolic BP, and the number of cigarettes smoked per day. The rationale for including these parameters in statistical analyses was to assess group differences independently of other established cardiovascular risk factors aside from hypertension. Because monocytes are the main source of cytokine production upon stimulation with LPS, whole blood cytokine production was corrected for the monocyte count.<sup>12</sup> In contrast, we did not correct basal TNF- $\alpha$  levels for monocyte count, as basal TNF- $\alpha$  levels likely originate from different kinds of cells and not only from circulating monocytes.

## Results

### Cardiovascular Risk Factors and Health Factors

Table 1 shows that hypertensive subjects were older and that they had higher BMI, HbA1c, and LDL/HDL ratio than normotensive subjects. Parents of subjects with hypertension had more often experienced cardiovascular events (number of myocardial infarctions and strokes combined) than parents of subjects with normal BP (odds ratio [OR] = 2.0, 95% confidence interval [CI] = 1.1 to 3.8,  $P = .028$ ). To ascertain that this effect was not confounded by the age of the study participants, we conducted multivariable logistic regression analysis on the most prominent effect, the difference in reporting that the participant's mother had experienced a stroke. After controlling for the participants' age, individuals with high BP at examination reported significantly more often that their mother had experienced stroke than individuals with normal BP (adjusted OR = 7.5, 95% CI = 1.4 to 40,  $P = .018$ ).

In subsequent analyses, we controlled for all cardiovascular risk factors to rule out their influences on the observed dependent variables. Of note, TNF- $\alpha$  was not different between normotensive and hypertensive individuals, and neither was there a significant correlation between systolic BP ( $r = -0.01$ ,  $P = .91$ ) and diastolic BP ( $r = 0.04$ ,  $P = .59$ ) with basal TNF- $\alpha$  in vivo. We found similar overnight urinary cortisol excretion in hypertensive and normotensive individuals, which may indicate



**FIG. 1.** Lipopolysaccharide (LPS)-stimulated release of tumor necrosis factor (TNF)-α in hypertensive compared with normotensive men (mean ± SEM). Hypertensive men had higher LPS-stimulated cytokine release than did normotensive men. \* $P = .024$ .

that subjects with differential LPS stimulation of TNF-α had equal activity of the hypothalamus–pituitary–adrenal axis.

### Stimulation of Cytokine Production

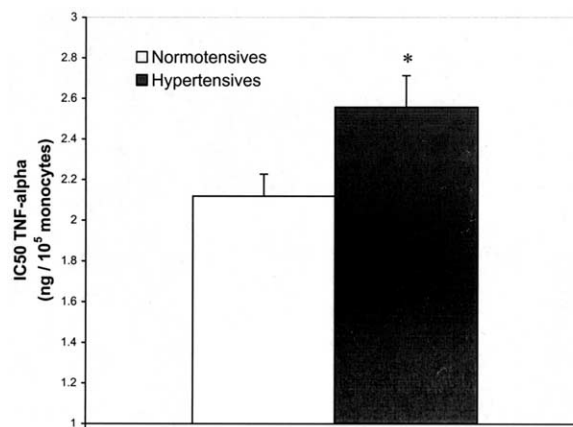
There was no significant association of systolic BP ( $r = -0.04$ ,  $P = .60$ ) or of diastolic BP ( $r = -0.02$ ,  $P = .82$ ) with change scores of TNF-α from baseline to LPS stimulation. After the absolute monocyte count and previously mentioned cardiovascular risk factors were taken into account, hypertensive subjects showed higher LPS-stimulated TNF-α release in vitro than normotensive subjects ( $F_{1,181} = 5.21$ ,  $P = .024$ ; Fig. 1). The absolute difference was about 11%.

### Glucocorticoid Sensitivity of Cytokine Release

Crude correlation analyses showed positive relationships between the  $IC_{50}$  and BP that reached significance for systolic BP ( $r = 0.14$ ,  $P = .044$ ) but not for diastolic BP ( $r = 0.11$ ,  $P = .125$ ). After controlling for the set of cardiovascular risk factors, hypertensive subjects had higher  $IC_{50}$  of TNF-α than normotensive subjects ( $F_{1,178} = 4.94$ ,  $P = .027$ ; Fig. 2). The absolute difference was about 21%. In other words, more dexamethasone was required to suppress TNF-α secretion in response to the same amount of LPS in hypertensive compared with normotensive individuals. This finding suggests that hypertensive men had lower monocyte glucocorticoid sensitivity than normotensive men.

### Discussion

The main finding of our study is that monocytes of hypertensive men required larger quantities of dexamethasone to inhibit LPS-stimulated release of TNF-α than did monocytes of normotensive men. In addition, LPS-stimulated TNF-α release per cell without glucocorticoid inhi-



**FIG. 2.** Glucocorticoid sensitivity of lipopolysaccharide (LPS)-stimulated release of tumor necrosis factor (TNF)-α between groups. The  $IC_{50}$  is inversely related to glucocorticoid sensitivity; ie, higher  $IC_{50}$  indicates lower glucocorticoid sensitivity and vice versa. Hypertensive subjects required relatively more dexamethasone to suppress LPS-stimulated TNF-α secretion than normotensive subjects (\* $P = .027$ ). In other words, monocytes of hypertensive men were less sensitive to dexamethasone suppression than monocytes of normotensive men.

bition was significantly higher in subjects with hypertension. Despite the observation that plasma levels of unstimulated TNF-α were not different between hypertensive and normotensive subjects, our findings suggest higher pro-inflammatory activity in blood monocytes of hypertensive men. This possibility is even stronger given that we controlled for classic cardiovascular risk factors, which were relatively more prominent in the hypertensive subjects.

What are the potential clinical implications of these findings and how do they compare with the literature? Our results indicate that the proinflammatory response of monocytes to an equal amount of LPS is higher in hypertensive than in normotensive men. Atherosclerosis is largely an inflammatory disease<sup>5</sup> in which TNF-α production by monocytes and macrophages plays a central role.<sup>29</sup> Although this has not been proved, several lines of evidence point to the possibility that hypertension is a contributor to the inflammatory response of the vessel wall.<sup>30</sup> Based on our findings, any LPS-like danger signal (such as one related to infection or to cellular stress) might elicit relatively more proinflammatory cytokine production in hypertensive men, thereby promoting atherosclerosis. Such reasoning is in line with epidemiologic data supporting the notion that elevated plasma levels of TNF-α increase the risk of coronary artery disease in nonhypertensive populations.<sup>10,11,25</sup> It must be emphasized, however, that our study was cross-sectional. We thus are unable to make the distinction whether hypertension causes an increase in cytokines and thereby atherosclerosis or whether atherosclerosis as a result, for example, of increased shear forces to the endothelium causing increased cytokine production. Prospectively designed studies are necessary to address this important issue.



Our observation corroborates a previous study that found increased monocyte TNF- $\alpha$  and IL-1 $\beta$  release after LPS stimulation in hypertensive subjects.<sup>15</sup> Like our study, that study was unable to resolve the issue of whether preactivated monocytes in hypertensive individuals are an epiphenomenon or a causal factor triggering hypertension or atherosclerosis or both. In another study, hypertensive persons showed increased IL-1 $\beta$  secretion after LPS stimulation, whereas TNF- $\alpha$  production capacity of monocytes was decreased.<sup>31</sup> The investigators explained this discrepancy by a differential regulation in the synthesis of the two cytokines.

To the best of our knowledge, impaired glucocorticoid sensitivity of LPS-stimulated monocyte cytokine release in hypertensive individuals has not previously been reported. We offer two possible biologic mechanisms that might underlie the impaired ability of dexamethasone to down-regulate LPS-stimulated TNF- $\alpha$  release in monocytes of hypertensive persons. First, a population-based study found that hypertensive men had higher morning plasma cortisol levels than normotensive men.<sup>32</sup> Although our hypertensive and normotensive subjects had similar overnight cortisol excretion, we cannot exclude the possibility of intermittent peaks of exaggerated cortisol secretion among hypertensive persons, perhaps resulting in down-regulation of their glucocorticoid receptors.<sup>33,34</sup> Second, cortisol binding to glucocorticoid receptors is impaired in hypertensive persons,<sup>35</sup> rendering a possible explanation for the observed blunted glucocorticoid sensitivity in our hypertensive subjects.<sup>34</sup>

Similar to our study, three previous studies found no differences in unstimulated (ie, basal) plasma TNF- $\alpha$  between hypertensive and normotensive subjects.<sup>31,36,37</sup> One possible explanation for unchanged basal TNF- $\alpha$  among hypertensive and normotensive subjects concurrent with exaggerated LPS-stimulated TNF- $\alpha$  release in hypertensive persons might relate to the stage of the hypertensive disease. Indeed, one study found slightly increased basal TNF- $\alpha$  only in hypertensive subjects with chronic renal failure as opposed to subjects having uncomplicated hypertension and normotensive control subjects.<sup>37</sup> Unfortunately, we did not assess renal function; we assume, however, that the vast majority of our middle-aged subjects had not yet developed hypertensive organ damage.

Aside from the cross-sectional nature of our study, which does not allow us to interpret the direction of the hypertension-cytokine-atherosclerosis link, three other limitations need to be addressed. First, our screening procedure to track subjects with clinical hypertension might embed a recruitment bias because we measured BP on one occasion only. Also, our study lacked a systematic assessment of end-organ damage, and the collection of health variables (including use of antihypertensive medication) was by subjects' self-report. Nonetheless, despite this seemingly unsophisticated BP assessment, hypertensive subjects reported significantly more often having a parent

who had experienced a major cardiovascular event, for which hypertension is a strong risk factor, than did subjects who were normotensive. This may suggest that our categorization of individuals in hypertensive and normotensive subjects was not too simplistic. Second, our investigation focused on a working male population and our findings may not be generalizable to women, clinical populations, and individuals with overt vascular disease. Third, the observed differences in monocyte proinflammatory activity between hypertensive and normotensive men were small. Only the longitudinal follow-up of our cohort, linking glucocorticoid sensitivity with cardiovascular endpoints, will show whether these differences are of clinical relevance.

In the meantime, the concept of allostasis and allostatic load may help to put our findings in a clinical perspective. Allostasis refers to physiologic adaptation to environmental challenges, and allostatic load designates health burden as a consequence of inefficient allostasis.<sup>38</sup> Increase in BP can be understood as an adaptive effort mediated by the hypothalamus-pituitary-adrenal axis and the sympathetic nervous system, to meet physiologic demands imposed, for example, by the work environment.<sup>39</sup> Exposure to hypertension often persists for many years before the condition is brought to medical attention and treatment. Numerous accompanying events stimulating monocytes might, on each occasion, result in a slightly more pronounced inflammatory response. This, in turn, may increase the likelihood that other physiologic processes such as glucose hemostasis or lipid metabolism may become deviant from the physiologic equilibrium. Such deviations across multiple physiologic systems are characteristic for the early stages of allostatic load, predicting biologic morbidity and mortality later on in life.<sup>40</sup>

In summary, our data suggest that middle-aged hypertensive men show reduced responsiveness of monocytes to glucocorticoids, implying relatively sustained cytokine production once monocytes have encountered a stressful stimulus. Such an exaggerated reactivity of monocytes to external stimuli and diminished curtailing of monocyte proinflammatory responses by glucocorticoids might represent mechanisms linking hypertension with poor cardiovascular outcome. The clinical implications of our observations in health and in cardiovascular disease remain to be demonstrated.

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